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Article

The diversity of ursodeoxycholic acid precursors from bile waste of commercially available fishes, poultry and livestock in Indonesia

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Ursodeoxycholic acid (UDCA), a secondary bile acid (BA), has been used as a drug to treat various liver diseases. UDCA is synthesised from cholic or chenodeoxycholic acid (CA/CDCA), two primary BAs frequently used as the starting materials. Nowadays, swine, cattle, and poultry bile are the main sources of those BAs. However, other commercial animals could be promising sources as well. We identified two livestock, two poultries, and eight fishes that are commercially cultivated in Indonesia. Four free BAs including CA, CDCA, deoxycholic acid (DCA), and lithocholic acid (LA) were identified for their occurrences using thin-layer chromatography and high-performance liquid chromatography. CA was detected in cow, duck, red tilapia, gourami, the common carp, and grouper, whereas CDCA was only detected in two poultries and the common carp. The occurrence of DCA was common and abundant in most tested animals. In contrast, the presence of LA was found to be very low in all samples. The biliary bile of tilapia has been found to contain a high abundance of free CA (43% of the total bile). A simple extraction was able to purify CA from biliary bile of tilapia. This is a new promising and competitive source of CA.

Keywords: Ursodeoxycholic acid. Bile acid. Liver diseases. Cholic acid. Tilapia.

INTRODUCTION

Ursodeoxycholic acid (UDCA, Figure 1) has been applied as a pharmaceutical ingredient for the treatment of various liver disorders. It was first reported that the administration of UDCA in patients with gallstones solubilised cholesterol gallstones (Makino *et al*., 1975). Its properties, such as hydrophilic and nontoxic BA, inhibit the intestinal cholesterol absorption and increase the conversion of cholesterol to bile acids which eventually reduce the cholesterol saturation in the bile. A number of studies show that UDCA improves liver function in patients with cholestatic diseases. Today, UDCA is the only drug approved by the United States Food and Drug Administration for the treatment of primary biliary cirrhosis (PBC). In addition, its efficacies are under clinical investigation for other cholestatic liver diseases such as primary sclerosing cholangitis, intrahepatic cholestatic of pregnancy, liver diseases in cystic fibrosis, progressive familial intrahepatic cholestasis, as well as for other non-cholestatic liver diseases such as chronic viral hepatitis, liver transplantation, and alcoholic liver disease. The daily optimal dose is reported to be 8 to 10 mg/kg (Eggert, Bakonyi, Hummel, 2014). The detail of the mechanism, pharmacological activities, and its current use in clinical practice are well reviewed in Wang and Wu (2017).

UDCA was first identified as the chemical constituent in the bile of bears which was thought to be responsible for the efficacy of the traditional Chinese drug "Yutan" derived from dried bile of adult bears. It was used to alleviate hepatobiliary disorders (Bachrach, Hofmann, 1982; Lazaridis, Gores, Lindor, 2001). However, this natural UDCA is very difficult to

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get since it can only be isolated from the bile of bears which is scarce, unsustainable, and not economically competitive. Though bear farming was introduced to meet the demand, the current number has dropped since the Chinese government began to regulate the process, prohibit the hunting of wild bears, stop giving licenses to new farms, and shut off rogue farms that cannot follow the regulation. In addition, the extraction of bile from living bears is considered to be extremely cruel, inhumanly and unethically method, and therefore becoming serious attention from the animal welfare of view (Li *et al*., 2016; Liu *et al*., 2017). As a substitution, UDCA is currently made through chemical synthesis by using cholic acid (CA) or chenodeoxycholic acid (CDCA) as starting materials (Figure 1). Both CA and CDCA are primary BAs abundantly found in bovine bile (Tonin, Arends, 2018). In addition, the use of other BAs (Figure 1), e.g. deoxycholic acid (DCA) and lithocholic acid (LA), as precursors of UDCA is now under investigation and seems to be promising since they might provide alternative and short chemical routes (Kollerov *et al*., 2013, 2016; Deshcherevskaya *et al*., 2016; Tonin, Arends, 2018). Currently, the commercial BAs are mainly obtained from biliary bile of swine, cattle, and poultry such as chicken, duck or goose (Kim *et al*., 2007).

The availability of BAs for precursors of UDCA is still a challenging process. The current demand for UDCA necessitates a supply of tons of biliary bile which can only be obtained from meat industries. The major industries are located in highly populated and newly industrialised countries such as India, Brazil, and China. Apparently, there might be inadequate protocols such as technical or hygienic conditions that should be taken into account (Tonin, Arends, 2018). Moreover, an emerging issue should be considered regarding the use of animal bile. To date, the expanding global Muslim population has increased the need for *halal* pharmaceuticals. The halal status of pharmaceutical ingredients guarantees the absence of pork and its related products, other non-permitted animals, or alcohols. Furthermore, the slaughters procedures for the permitted animals should be performed according to the halal method (Islamic law). In parallel to the halal method, for Jews, the animals should be processed by following the kosher method (Jewish ritual) (Farouk *et al*., 2014; Norazmi, Lim, 2015).

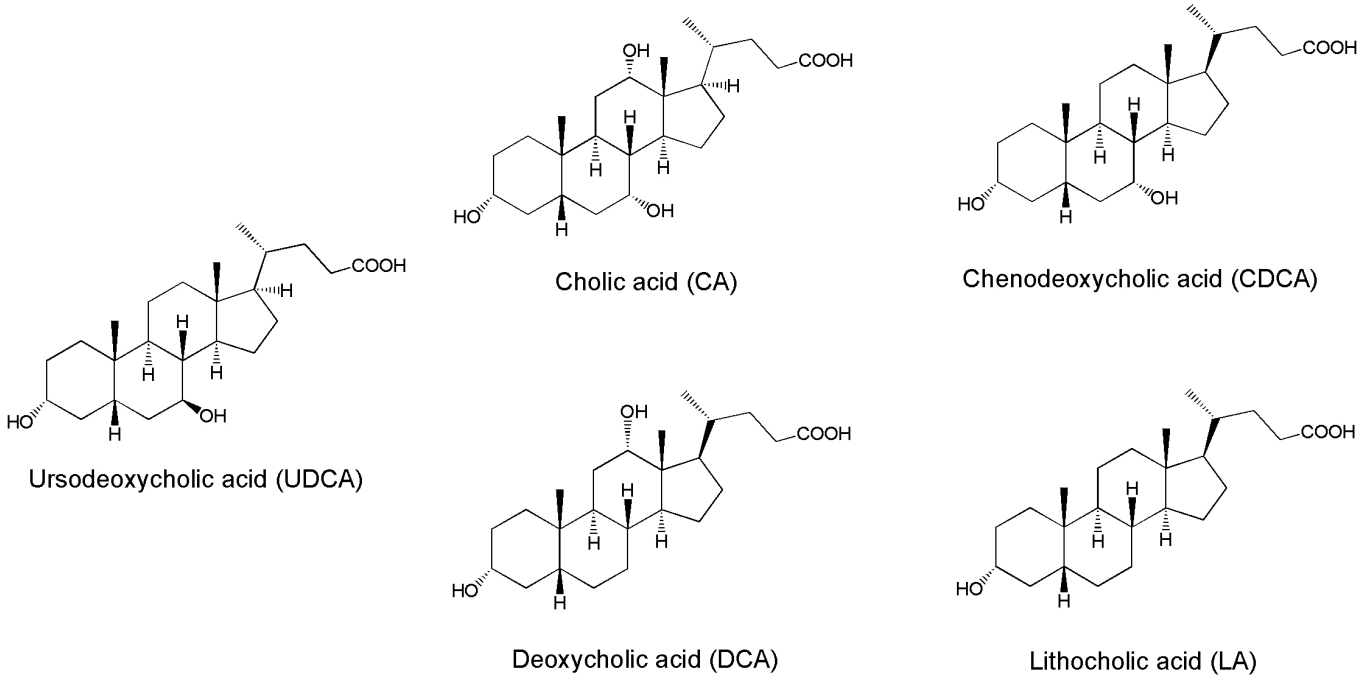

FIGURE 1 - Chemical Structure of Bile Acids.

As the country where the world biggest inhabitant of Muslims are living, the Indonesian government, as well as the citizens, are highly aware of the halal status of food and pharmaceutical ingredients. The government has been pursuing to regulate halal products by founding the Indonesian halal certification authority. Accordingly, the need of BAs from halal animals as precursors of UDCA is required if this drug will be produced and used in Indonesia or in other Islamic countries. In this work, we reported profiles of four bile acids related to UDCA precursors from several permitted animals cultivated in Indonesia. Although poultry and cattle bile are currently the main sources, other commercial animals, e.g. fish, could be promising sources for BAs as well.

MATERIAL AND METHODS

Chemicals and reagents

Standard of BAs including cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and lithocholic acid (LA), were purchased from Sigma-Aldrich. Other chemicals i.e. methanol (p.a., LC grade), acetonitrile (LC grade), ethanol (p.a.), ethyl acetate (p.a.), *n*-hexane (p.a.), chloroform (p.a.), phosphomolybdic acid (p.a.), TLC silica gel 60 F_{254} (aluminium plate, 20 x 20 cm), acetic acid (p.a.), sulphuric acid (p.a.), ammonium carbonate (p.a.), etc. were obtained from Merck (Darmstadt, Germany).

Bile sources

Biliary bile animals were collected from the numbers of fresh fishes including *Cyprinus carpio* (the common carp), *Osphronemus goramy* (the giant gourami), *Oreochromis niloticus* (red tilapia), *Brama brama* (pomfret), *Epinephelus coioides* (orange-spotted grouper), *Clarias spp.* ("sangkuriang" Indonesian catfish), *Channa striata* (striped snakehead), *Pangasius spp.* (Indonesian pangasius), two poultries (chicken and duck), and two livestock (cattle and sheep). Tilapia, orange-spotted grouper, pomfret, and catfish were obtained from Balai Layanan Usaha Produksi Perikanan Budidaya Karawang – Ministry of Marine Affairs and Fisheries Republic of Indonesia. Carps, gourami, striped snakehead, and pangasius were obtained from local markets. Poultry and livestock were obtained from local slaughterhouses.

Extraction of BAs

Fresh bile of animals was dried in an oven at 60 ºC until a constant weight was achieved. Samples of dried bile (0.1 to 1 g) were extracted twice through ultra-sonication with 10 mL methanol at 50 ºC for 15 min with vigorous mixing in between. Extracts were filtered and concentrated under reduced pressure to obtain dried bile extracts for the analysis and further experiments.

Analysis of BAs

Thin-layer chromatography (TLC)

TLC analysis was performed with a normal phase silica gel 60 F254 (Aluminium plate, 20 x 20 cm, Merck). An applicator, Linomat 5 (CAMAG) combined with 100 µL Linomat Syringe was used with the help of nitrogen air. The application of samples was set as follow: 6 mm of spot band, 11 mm of the interval between spots, and 10 µL of volume injection. Chromatography was performed in a normal developing chamber that has been equilibrated with a mobile phase consisting of chloroform-ethyl acetate-acetic acid, 45:45:7 (v/v) for one hour. After developing the plate using the mobile phase up to 8 cm from the initial spot, the plates were air dried and sprayed with 10% phosphomolybdic acid in 96% ethanol (w/v) and heated with a heat gun 2 to 3 min until coloured zones appeared. The BAs will appear as bluish black spots under visible light (Wollenweber, Kottke, Owen, 1966).

High-performance liquid chromatography (HPLC)

The bile components were analysed by using LC-20AD liquid chromatography equipped with SPD-20A UV/Vis detector and CTO-20A oven pump (Shimadzu, Japan). A reversed-phase column, LiChrospher® 100 RP-18 5 µm column (100 mm length, 4 mm diameter, 20 mm pre-column, Merck), was used for separation. The HPLC condition was as follows: the mobile phase consisted of 35% (v/v) acetonitrile with 0.3% (w/v) ammonium carbonate, the column temperature was set to 30 ºC, the flow rate was 0.5 mL/min, volume injection was 20 µL, and UV wavelength was 202 nm. Exceptionally, for LA analysis, the composition of acetonitrile was increased to 60% (v/v). The linearity of the HPLC system for each standard of BAs as well as the limit of detection and quantification, LOD and LOQ,

were determined according to the method described in Baalbaki *et al*. (2018) and Soo-Lim *et al*. (2018).

Liquid chromatography-tandem mass spectrometry (LC-MS)

LC-MS analysis was performed on UPLC-Waters coupled with XEVO-QTOFMS with an ESI source. The separation was carried out on an XSelect CSH C18 column (2.1 x 100 mm, 5 µm) applying a linear gradient of 30 to 98% acetonitrile in water, both containing 0.1% formic acid, running time in 15 min, column temperature 30 ºC, and flow rate at 0.2 mL/min. The parameter was set as following: electrospray negative ion mode for ionisation, capillary voltage with 3.5 kV, and collision energy with 50 eV.

RESULTS

Selected animals and their biliary bile profiles

Two livestock, two poultries and eight fishes were used as bile sources in this experiment. The animals selected were not only commercially available but also can be cultivated in huge number to guarantee the continuous supply when used as sources of pharmaceutical ingredients. Table I describes the animal samples used and their biliary contents. The total weight of biliary bile for a single cow, due to its size, is larger than other selected animals. Surprisingly, although the size of sheep is the second after the cow, one sheep only has about 3.87 g of biliary bile. In poultries, where chicken or duck has the average size of 1 to 1.3 kg, the weight of biliary bile is around 2 gram per poultry. In contrast, the biliary bile contents found in several fishes are larger than in poultry. One kilogram of fish consists of up to 11.6 g of bile. Among eight fishes, red tilapia, gourami and common carp have a bigger size of the gallbladder and therefore contribute to the higher bile content. In general, water constitutes more than 80% of the biliary bile content. Methanol extraction of the dried bile yielded up to 90% of BA paste in most selected animals, around 60% in fewer animals. The Indonesian pangasius has the lowest yield of BA paste which was only 1.87% of the dried bile.

TABLE I - Animal samples and their biliary bile contents

Sample	Unit of sample	Bovine bile			Yield of bile acid paste*, % (w/w)
		Fresh weight (FW), g/unit of sample	Dry weight (DW), g/unit of sample	Water content, % (w/w)	
Cow	cow	255.46±6.93[a]	20.97±3.27	91.79±1.32	90
Sheep	sheep	3.87±0.63	0.49±0.05	87.23±2.70	60
Chicken	chicken	2.03±0.15	0.19±0.06	90.48±3.04	89
Duck	duck	2.05±0.15	0.10±0.02	95.07±1.16	74
Red tilapia	kg	11.66±0.77	1.54±0.2	86.79±1.38	96
The giant gourami	kg	4.76±0.13	0.45±0.01	93.52±2.76	98
Common carp	kg	9.16±0.61	1.44±0.18	84.28±4.05	85
Striped snakehead	kg	3.75	0.57	84.8	53

(Continuing)

TABLE I - Animal samples and their biliary bile contents

Sample	Unit of sample	Bovine bile			Yield of bile acid paste*, % (w/w)
		Fresh weight (FW), g/unit of sample	Dry weight (DW), g/unit of sample	Water content, % (w/w)	
Catfish "sangkuriang"	kg	2.18	0.41	81.19	96
Indonesian pangasius	kg	2	0.24	88	1.87
Pomfret	kg	3.27	0.64	80.43	61
Orange-spotted grouper	kg	3.25	0.37	88.61	49

* Paste as the total of methanol extract per dried bile
[a] Data of mean and SD were at least triplicate of unit

Chromatographic analysis of bile acids

Thin-layer chromatography (TLC) has been applied for the separation and analysis of a wide variety of BAs. Not only simple and inexpensive, but it can also separate BA components into free and conjugated forms albeit the resolution was generally unsatisfactory (Scalia *et al*., 1994). In this study, TLC analysis was chosen for the routine analysis. This is the easiest and quickest way to study the profile of BAs from multiple sources. Moreover, it can be used to follow every step during the extraction and purification of bile components. A typical TLC profile of four bile acids was shown in Figure 2, where CA, CDCA, DCA, and LA were well separated in an 8-cm distance of the silica plate with the good shape of spots. In addition, the spray reagent used provided bile spots which were contrast, very specific, and long-lasting colours, compared to other reagents (e.g. Liebermann-Burchard).

Although the TLC method is good enough to identify the targeted BAs, the more sensitive of quantitative analysis using other advanced methods is necessary. Analysis with HPLC will help to obtain better separation and to quantify the bile components precisely. A number of studies have reported various conditions for HPLC analysis of BAs. In this work, an established HPLC method from Yeh and Hwang (2001) was modified slightly. Figure 3 shows the retention time of the four authentic standards, CA, CDCA, DCA, and LA. It suggests that the modified method was good enough to separate the BA components. CA, CDCA, and DCA can be analysed simultaneously by a single mobile phase consisting of 35% (v/v) acetonitrile with 0.3% (w/v) ammonium carbonate, while LA, though it was detected around 17 min of the retention time, the peak was not sharp and symmetry (data not shown). Therefore, for LA, the second mobile phase consisting of 60% (v/v) acetonitrile with 0.3% (w/v) ammonium carbonate was used and resulted in the good peak around 5 min of the retention time.

Table II shows the quantitative conditions of four BAs by using HPLC method. This method was capable of differing four bile acids through their retention time, in particular, the separation of CDCA and DCA was well achieved compared to the TLC method. The linear range of four BAs was achieved at 2.5 µg to 100 µg (it is equal to 125 µg/mL to 5000 µg/mL), where the correlation coefficient was near one. Both CA and CDCA have similar LOD and LOQ at 0.6 and 2 µg, DCA at 0.45 and 1.49 µg, respectively. LA has the lowest LOD and LOQ, 0.13 and 0.44 µg, respectively.

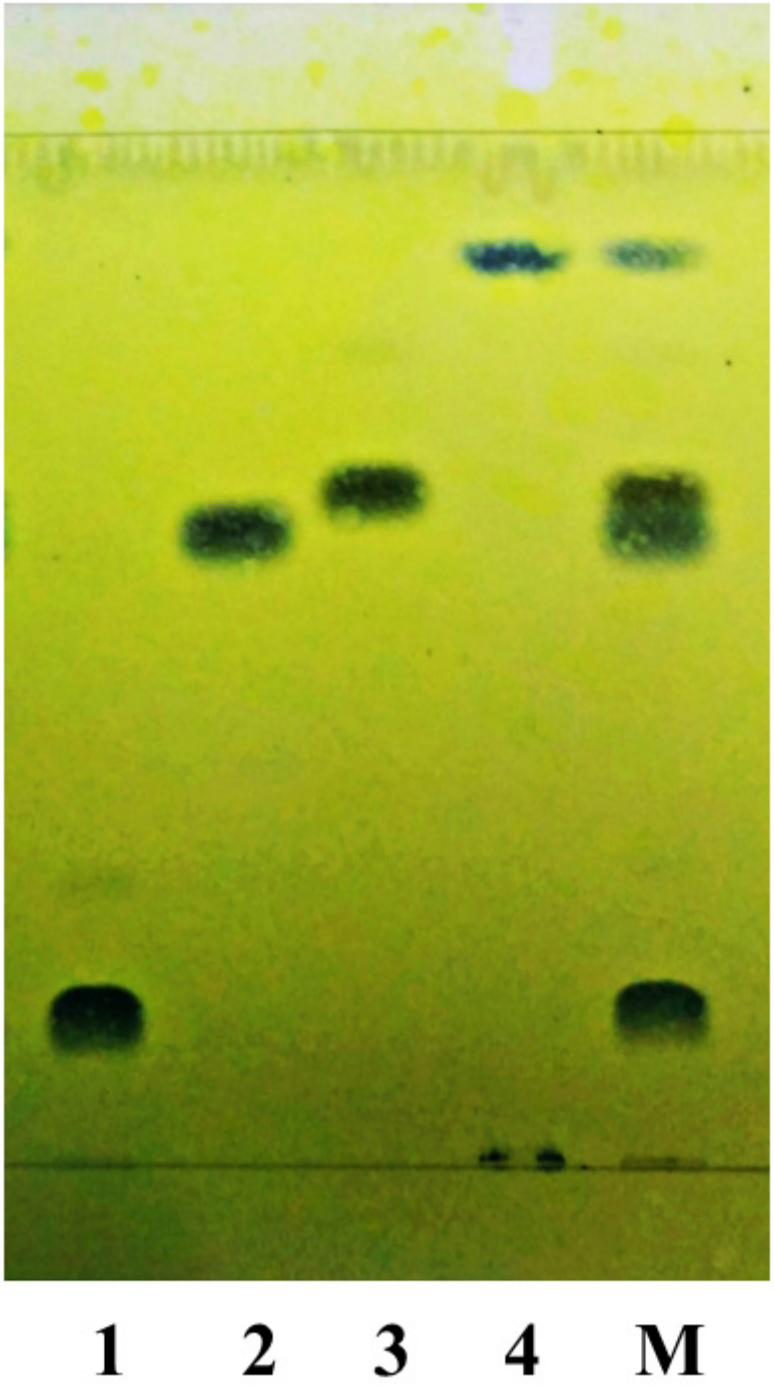

FIGURE 2 - TLC separation of the authentic BAs. Standards of CA (1), CDCA (2), DCA (3) and LA (4) were set to be 1 mg/mL. Each of standard and their mix (M) were dissolved in methanol and spotted for 10 µL onto silica plates with the help of Linomat 5.0.

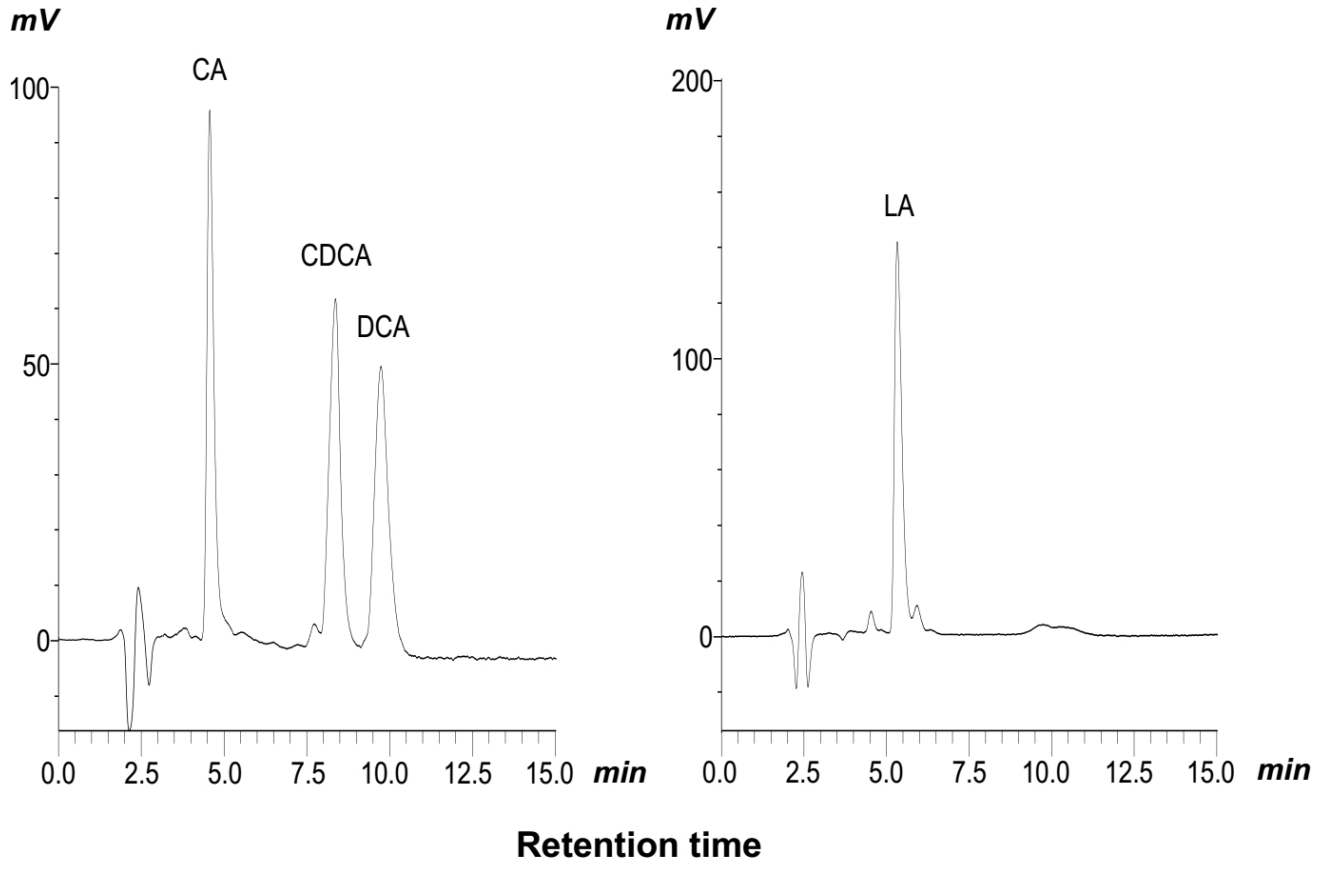

FIGURE 3 - HPLC separation of the authentic BAs. Twenty microliters of each standard (2500 μg/mL) were injected into the column. LiChrospherÆ 100 RP-18 5 μm column (100 x 4 mm), 20 mm of pre-column, 30 ºC of column temperature, 0.5 mL/min of flow rate, UV at 202 nm, 20 μL of injection. The concentration of each BA was 2500 μg/mL.

TABLE II - Optimised HPLC parameters of four free BAs

BAs	RT, min	LR, ug	R^2	LOD, ug	LOQ, ug
CA	4.48	5 - 100	0.9983	0.66	2.21
CDCA	8.14	5 - 75	0.9982	0.63	2.10
DCA	9.73	2.5-100	0.9997	0.45	1.49
LA	5.33	2.5 - 100	0.9994	0.13	0.44

RT, retention time; LR, linear range; R2, correlation coefficient; LOD, limit of detection; LOQ, limit of quantification.

Biliary bile components of the selected animals

Profiles of BAs from the selected animals were easily visualised with the TLC method (Figure 4). Accordingly, each of the animals has a certain pattern of biliary BAs. The prominent BA, CA, seemed to be present in most samples. The occurrence of CDCA was only well observed in chicken and duck, whereas the presence of DCA and LA was barely detected in all samples. In this work, however, the occurrence of glycine/taurine-conjugated BAs were not evaluated. An interesting pattern has been shown in two freshwater fishes, red tilapia and the giant gourami, in which a massive spot has been detected in both fishes. The spot could be CA or its conjugates. To validate the TLC result, the same bile samples were further analysed by HPLC method.

HPLC profiles and the weight of each BA component from the selected animals are shown in Figure 5-6 and Table III. In contrast to the TLC result, validation with the HPLC method confirms that CA is only detected in cow, duck, tilapia, gourami, the common carp, and grouper. The presence of CA in other animals could be as its glycine or taurine conjugates which might be indicated by the peak embedded or next to peak of CA. The HPLC method also confirms that the massive spot present in tilapia (*see* Figure 4) is CA which constitutes about 43% of the dry weight or 438 mg CA in one gram of dried bile. In gourami, however, the total CA was only 3% of the dry weight, suggesting that the massive spot in the TLC was not from the CA alone but could be in combination with its conjugate as confirmed by the presence of an embedded peak in the HPLC chromatogram. Moreover, in comparison to the TLC, the HPLC method was able to separate and identify biliary bile components present in the common carp.

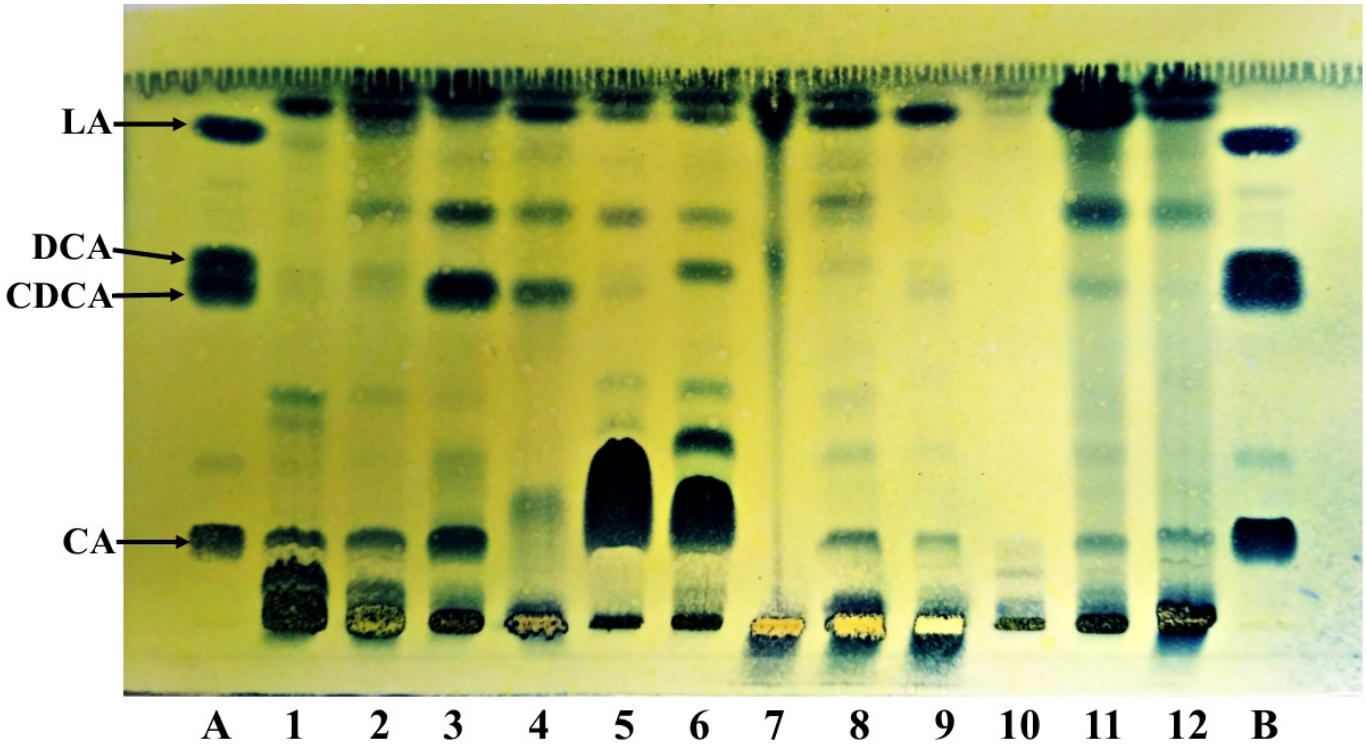

FIGURE 4 - TLC profile of biliary BAs from the selected animals. Standards of CA, CDCA, DCA and LA were set to be 1 mg/mL (A) and 2.5 mg/mL (B). Samples of 1 to 12 were cow, sheep, chicken, duck, red tilapia, gourami, common carp, striped snakehead, catfish, pangasius, pom-fret, orange-spotted grouper, respectively. Concentrations of samples were 25 mg/mL (3, 5, 6), 50 mg/mL (1, 2, 4, 7, 11), and 75 mg/mL (8, 9, 10, 12). Both standards and samples were dissolved in methanol and spotted for 10 μL onto silica plates with the help of Linomat 5.0.

The occurrence of CDCA was not common and only observed in the bile of chicken, duck and the common carp, which constitutes 3.3, 0.12, and 0.04% of the dried bile, respectively. Similarly, the presence of LA was found in a trace amount and only well observed in the biliary bile of sheep, duck, gourami, and grouper. DCA is mostly present in all tested bile, except in tilapia and gourami bile. This secondary BA was found to constitute most of the bile components in chicken, striped snakehead, catfish, and grouper which is up to 79, 46, 49, and 85% of the dried bile, respectively.

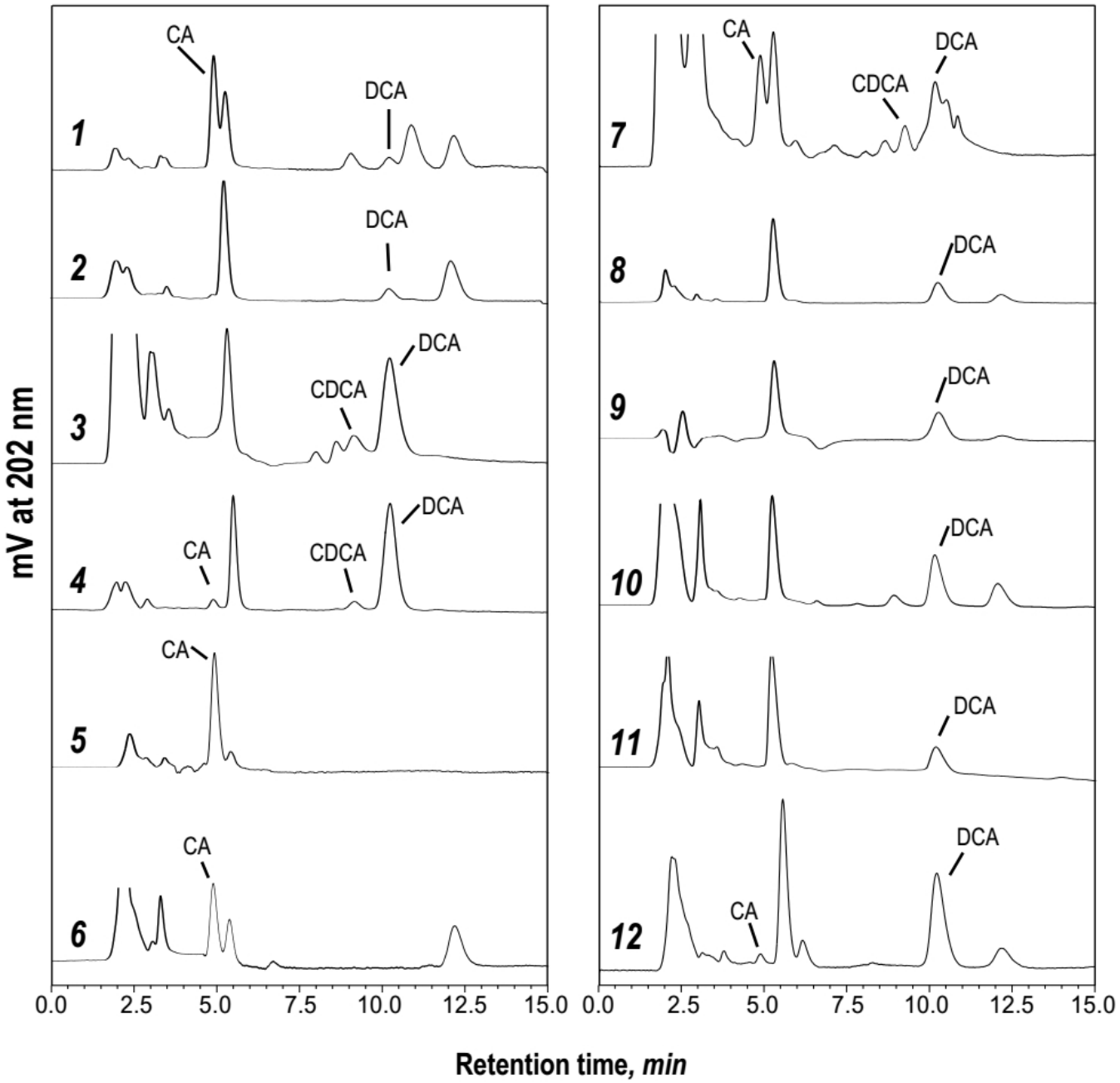

FIGURE 5 - HPLC profile of CA, CDCA and DCA from the biliary bile of the selected animals. Samples of 1 to 12 were cow, sheep, chicken, duck, red tilapia, gourami, common carp, striped snakehead, catfish, pangasius, pomfret, orange-spotted grouper, respectively. Concentrations of samples were 1 mg/mL (1, 2, 4, 5, 6, 8, 9, 12), 5 mg/mL (10, 11), and 10 mg/mL (3, 7).

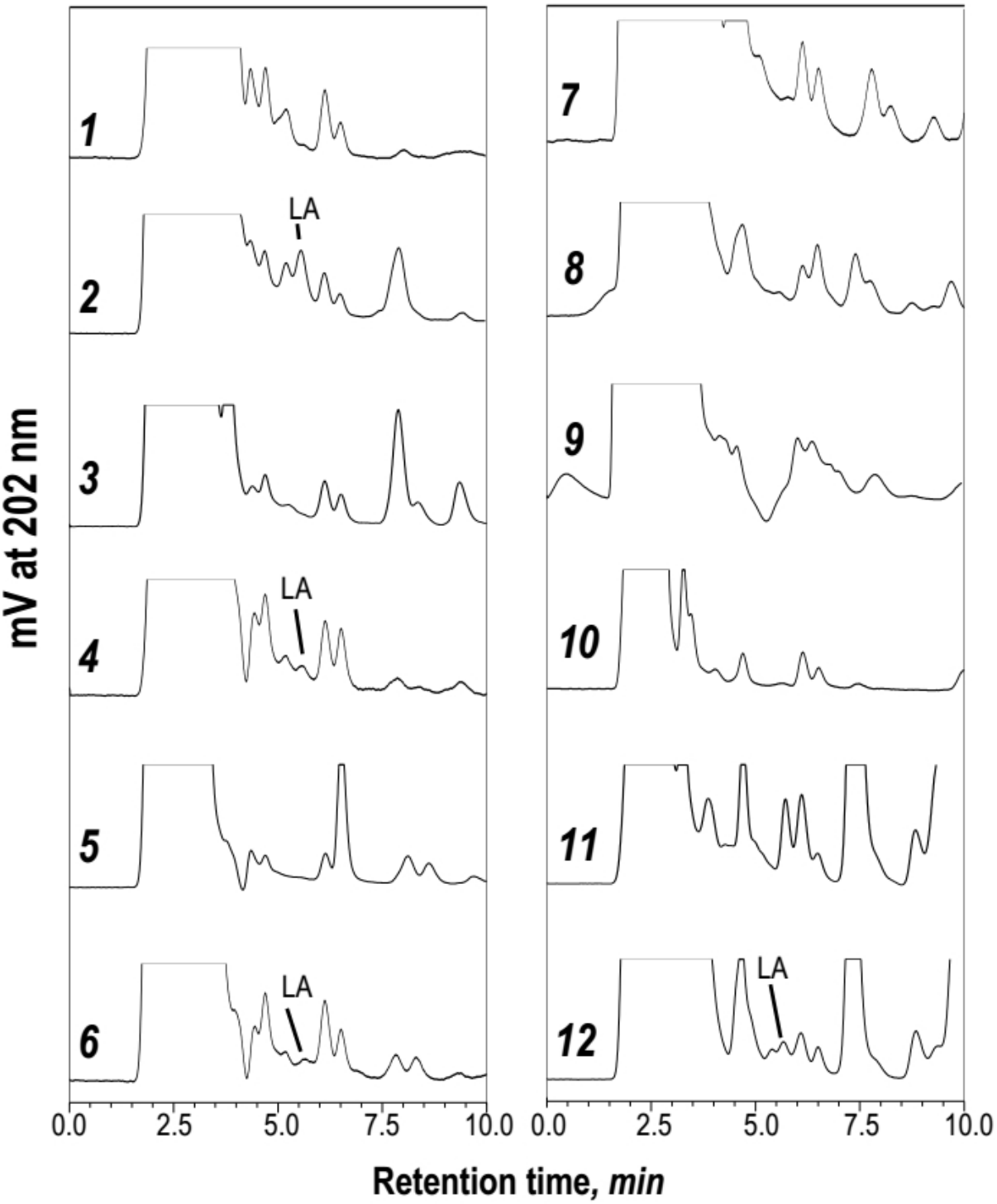

FIGURE 6 - HPLC profile of LA from the biliary bile of the selected animals. Samples of 1 to 12 were cow, sheep, chicken, duck, red tilapia, gourami, common carp, striped snakehead, catfish, pangasius, pomfret, orange-spotted grouper, respectively. The concentration of all samples were set to be 10 mg/mL.

TABLE III - Biliary BA composition of the selected animals

Sample	Concentration of BAs, mg/g [a]			
	CA	CDCA	DCA	LA
Cow	317.38±68.24[b]	n.d[c]	168.64±11.24	Trace
Sheep	30.77±5.13	n.d	99.95±3.06	27.83±3.29
Chicken	4.87±0.53	33.56±5.08	796.51±57.87	n.d
Duck	97.35±7.76	1.22±0.31	38.92±4.28	2.69±0.13
Red tilapia	438.81±33.98	n.d	n.d	n.d
The giant gourami	31.51±4.02	n.d	n.d	3.55±0.42
Common carp	51.82±5.45	4.40±0.50	35.37±6.14	Trace
Striped snakehead	trace[d]	n.d	469.97±40.18	Trace
Catfish "sangkuriang"	trace	trace	491.07±36.66	trace
Indonesian pangasius	trace	n.d	8.54±0.43	trace
Pomfret	trace	trace	8.66±0.61	trace
Orange-spotted grouper	n.d	n.d	854.78±41.20	6.60±0.55

[a] mg of BAs per gram of the dried bile (dry weight)
[b] Data represent mean±SD. Mean of triplicate
[c] Not detected
[d] Under limit of quantification

Purification of CA from tilapia bile: a preliminary study

By observing the complexity of BAs in tilapia, a simple purification has been applied to isolate the major CA. This present objective was not intended to completely purify all CA from bile extract of tilapia, but rather a preliminary test of systems that could be very useful in the developing of an ultimate extraction of free CA from tilapia bile. In this work, the purification steps including methanol extraction of dried bile (de-protein), phase separation with hexane (removing non-polar compounds), phase separation with ethyl acetate (CA move to ethyl acetate), washing, de-colourisation, and precipitation were used. Figure 7 shows the steps of the isolation of CA from tilapia bile.

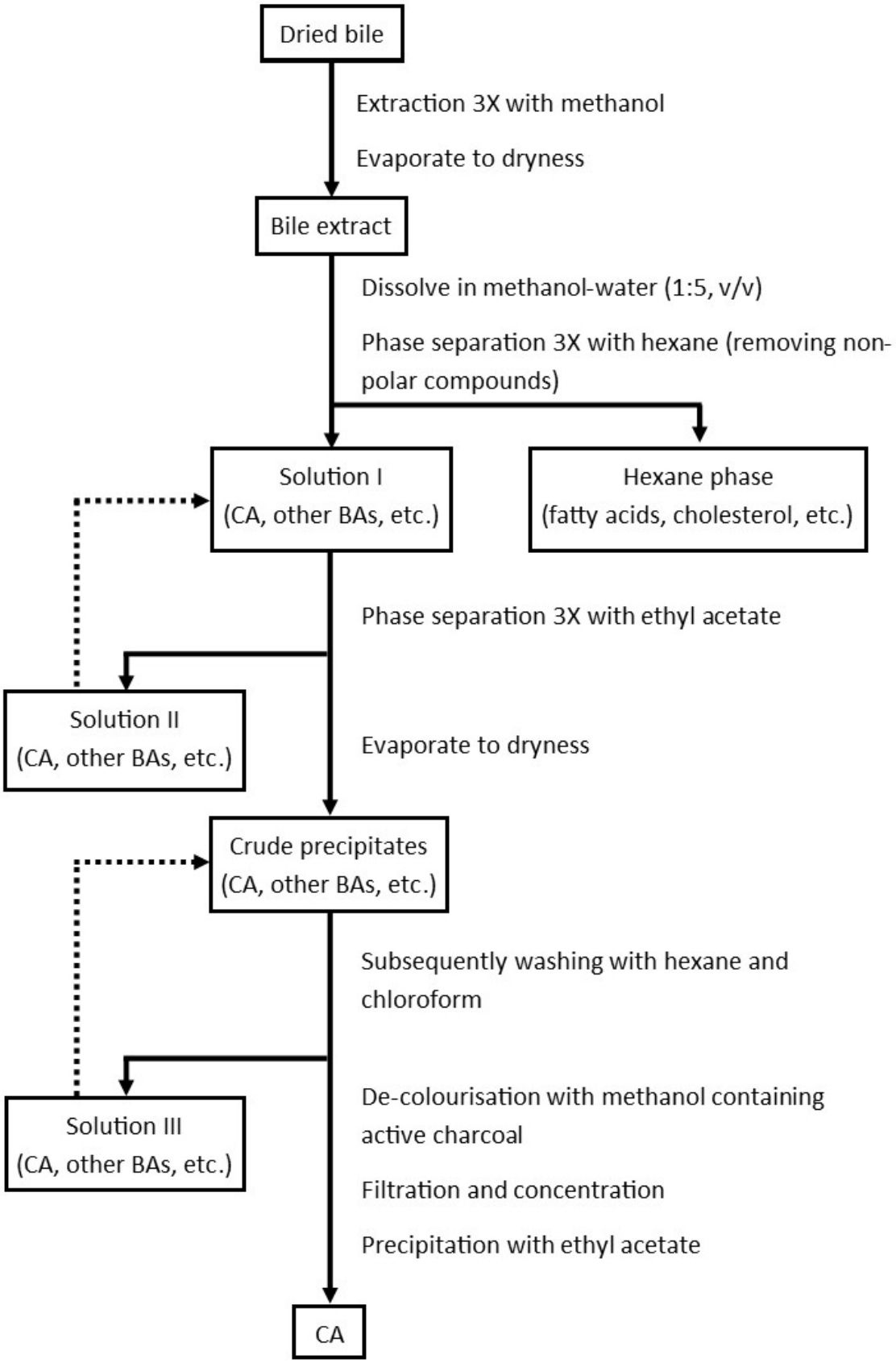

FIGURE 7 - Purification steps of CA from tilapia bile.

To our results, removing non-polar compounds of 8.97 g of tilapia bile extract with hexane removes only 330 mg thereof, which was about 3.67% (w/w) of the total bile extract. Such result indicates that the tilapia bile only contains small portions of fatty acids, cholesterol, or other highly non-polar substances, which is far lower than the other commercial sources, e.g. chicken or cattle bile (our unpublished results). Extraction of the residue (a solution I) containing a crude mixture of CA, other BAs, pigments, etc. with ethyl acetate can obtain crude CA as the precipitate. The crude CA obtained from phase separation with ethyl acetate was easily precipitated just after the exceed solvent evaporated. The crude precipitate was then washed with hexane and chloroform to remove the impurities. These solvents, however, also solubilised a small quantity of CA. Therefore, the removed parts should be re-crystallised again to recover the loss of CA. De-colourisation with active charcoal was able to remove most of the pigments resulting in highly pure CA. The purity of CA was analysed by using HPLC and MS analysis confirmed the exact mass of CA that produced a parent ion $[M-H]^-$ at *m/z* 407.3 in the negative ion mode (Figure 8).

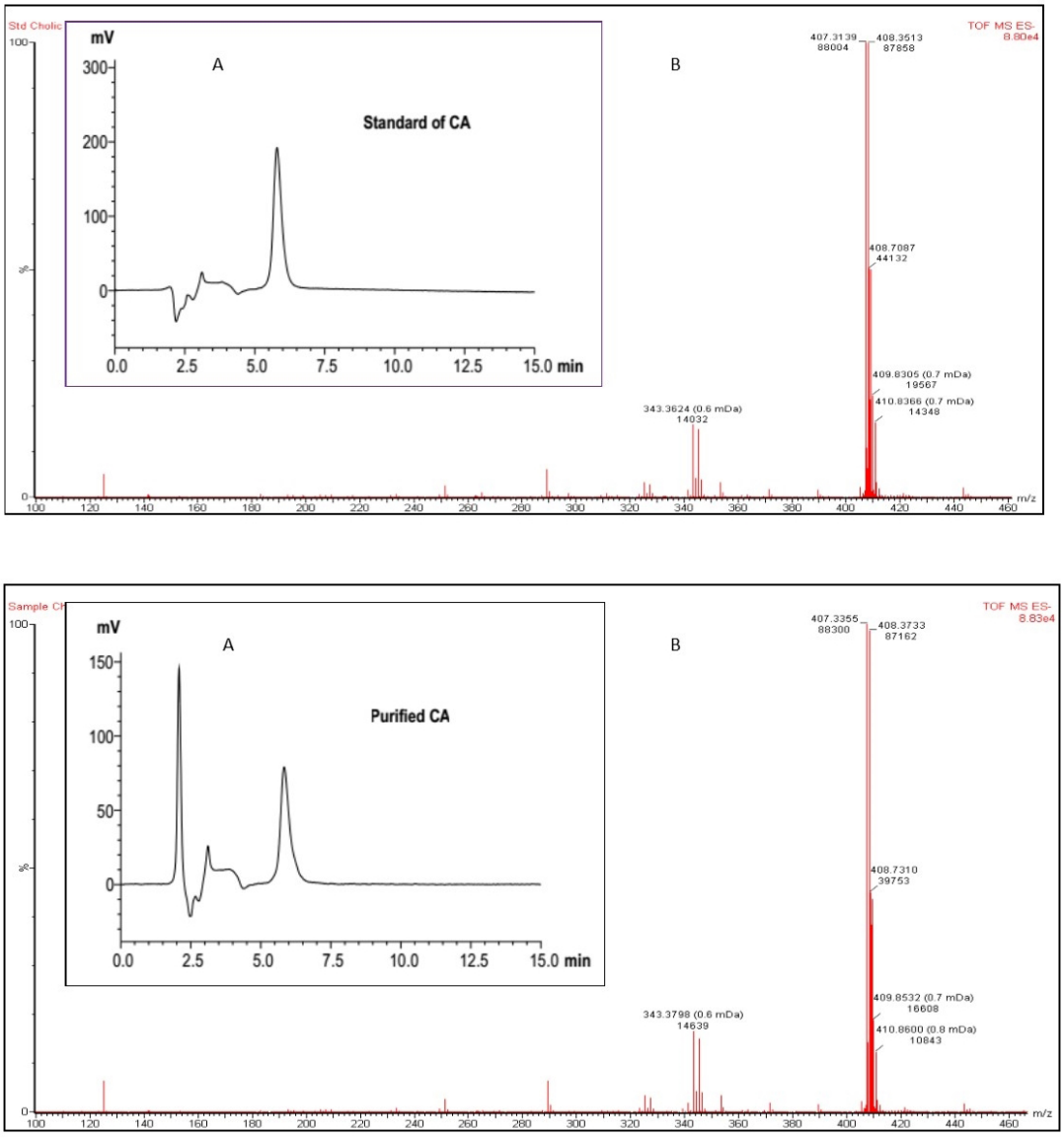

FIGURE 8 - Confirmation of the purity of CA with the HPLC (A) and product ion scan of CA in negative ion mode resulting in a parent ion [M-H]- at m/z 407.3 and one of daughter ions at m/z 343.3 (B). For the HPLC, the mobile phase consisted of 30% acetonitrile containing 0.3% ammonium carbonate. Samples were CA standard (upper) and the isolated CA (bottom).

DISCUSSION

UDCA drug is currently synthesised from CA or CDCA. The chemical routes are started by modification of the CA structure which involves protection and de-protection steps leading to the formation of CDCA. The process takes seven steps. Afterwards, CDCA is then converted to UDCA in two steps. Such process results in about 30% overall yield (Hofmann *et al*., 1963; Tonin, Arends, 2018). However, a recent report from He and co-workers shows that by eliminating and optimising several steps, UDCA can be produced from CA up to 65% overall yield with only seven steps (He *et al*., 2018).

The use of CA as the most appropriate precursor has been common due to its occurrence in most biliary bile of animals and can be extracted in good yields. It is consistent with the results reported here. Currently, bovine bile from cattle is the cheapest raw material and available in large quantity (Scalia *et al*., 1994; Tonin, Arends, 2018). It contains high quantities of CA and DCA but trace amounts of CDCA and LA, which are also confirmed in this experiment. However, CA of that bovine bile is mostly present in a conjugated form (Watanabe, Tsuneyama, 2012; Hu, Feng, Zhang, 2018) and therefore need to be de-conjugated to enhance the yield of free CA. This process uses a large quantity of alkaline, high temperature, and for extremely long times (Tonin, Arends, 2018) which eventually makes the process consumes higher cost and not environmentally friendly.

Of all tested animals, biliary bile of red tilapia fish contains the highest level of CA, which occurs mostly in its unconjugated form. Normally, BAs of fishes and other vertebrates are reported to be present largely in its conjugated forms, except in soda cichlid and Mozambique tilapia fishes in which major fraction of BAs are present in the unconjugated form (Hofmann, Hagey, Krasowski, 2010). Interestingly, CA constitutes up to 43% of BAs in tilapia and, in the meantime, the other three BAs are under detectable level. It seems that the red tilapia fish could be a new promising source of CA since the level of CA is far higher compared to the bile of cows or oxen (Watanabe, Tsuneyama, 2012; Hu *et al*., 2018). Moreover, considerations of the complexity of BA components and the individual yield of biliary bile support that idea. The composition of BAs in tilapia is less complex than in cattle bile. Therefore, a few steps of extraction such as de-conjugation and multi-steps of purification are not necessary which eventually reduce time and cost. The high individual yield of cow's biliary bile has made this bovine bile as the prominent source of CA. Our result, however, showed that the same amount of dried bile from a single cow could be compensated by 13 kg of tilapia fish. This is even more promising since the cultivation of tilapia relatively needs small efforts, a little space, and a very short harvest time (2 to 6 months) (Haque *et al*., 2016; Antwi *et al*., 2017; David, Pinho, Garcia, 2018).

A simple extraction has been preliminarily tested to purify free CA from tilapia bile. However, during the phase separation with ethyl acetate, this step was not able to completely recover the major part of CA from crude bile extract, even after ten times of ethyl acetate extraction. Of the 8.97 g of bile, about 500 mg overall yield of pure CA was obtained. Most of CA were not extractable. This can be explained by the natural behaviour of CA or BAs in general. They have been known for their hydrophilic and hydrophobic properties leading to the unusual solubilising capabilities which support their pivotal role during the absorption of various lipid substances from the intestine into the bloodstream. Any attempt to separate the bile components without modifying their properties such as hydrolysis, treatment with alkaline or acidification, is therefore considerably difficult (Ahrens, Craig, 1952). Accordingly, such modifications are needed to alter the partition ratio of bile components in order to easily purify CA from tilapia bile in good yields.

Biliary bile of chicken and duck have been known for their high CDCA content and therefore, used as sources of CDCA. Our results show that those poultries do have CDCA which consistent with the previous reports (Yeh, Hwang, 2001; Wan, He, Cao, 2012). Synthesis of UDCA from CDCA precursor provides short chemical routes and therefore is more preferably (Tonin, Arends, 2018). However, the purification of CDCA from the biliary bile is still a challenging process due to the bile complexity, the need of de-conjugation, and the yield which is lower compared to the yield of CA extracted from cattle bile (Ziegler, Attwell, Vergottini, 1980; Kim *et al*., 2007; Wan, He, Cao, 2012). Moreover, in Indonesia, most of the chicken slaughterhouses are decentralised as small or traditional slaughterhouses, making the collection of chicken bile uncompetitive. In addition, the consumption of duck is still far beyond the chicken which indicates that duck bile cannot meet the current demand (USAID, 2013; Statistics of Indonesia, 2018).

Other BAs, e.g., DCA and LA, are known as by-products during the extraction of multi-tons of bile

waste which have no real application for them yet. They are simply destroyed after separation from the valuable BAs and may enter the environment (Kollerov *et al*., 2013). DCA is the most abundant by-products of biliary bile which is also confirmed in our results. This BA is hepatotoxic, gastric damaging, and hazardous for the environment (Mahato, Mukherjee, Banerjee, 1994; Scalia *et al*., 1994). Unlike DCA, LA is present as the minor BA in most of the selected animals, except in sheep which is up to 2.7% of the dried bile. Recent reports showed that both DCA and LA could be promising precursors for UDCA in the future. The high abundance of DCA usually found in the biliary bile of animals can be turned into valuable derivatives of BAs by means of microbial transformation (Deshcherevskaya *et al*., 2016; Kollerov *et al*., 2016). Such derivatives might provide alternative pathways for synthesising of UDCA. Moreover, the most interesting result has been showed by microbial transformation of LA which only needs one step via hydroxylation at position 7β leading to the direct formation of UDCA. This is the shortest chemical routes for the synthesis of UDCA (Kollerov *et al*., 2013; Tonin, Arends, 2018). However, the low yields of LA found in the biliary bile of animals might become a limitation, particularly when using LA as the sole starting material for commercial production of UDCA.

CONCLUSION

Many attempts are recently pursuing to find alternative chemical routes for the synthesising of UDCA from CA or other BAs which are more competitive and environmentally friendly. Searching for alternative BA sources, however, was rarely reported. In this work, the diversities of the BAs that could be used as alternative sources are reported from several animals commercially cultivated in Indonesia. The occurrence of CA and DCA are abundant in most animals, CDCA is present only in poultries and the common carp, whereas LA occurs rarely and in very low yield. The red tilapia bile could be an alternative source of CA. It contains up to 43% of free CA and the bile composition is less complex than other readily commercial bile sources. A simple extraction was able but not completely to purify all free CA from the biliary bile of tilapia and therefore need to be improved in the future study. According to the individual yield, the same amount of CA from one cow can be compensated with 13 kg of tilapia fish (about 40 individual of tilapia). Moreover, the cultivation of tilapia only needs small efforts, a little space, and a very short harvest time, which make this alternative source of CA is worth to be considered.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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